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14. ABSTRACT

The focus of this program is the development of a pre-clinical blood-based TSE diagnostic. The assay is been developed with plasma from hamsters infected with the 263K strain of scrapie. The assay has shown high sensitivity and specificity and good reproducibility. In this funding period we addressed two critical issues: Proteinase K (PK) digestion to discriminate PrPc, the normal form of the protein and PrPres the infection-specific form of the protein and denaturation of PrPres in plasma. The first issue was partially resolved when we discovered that PK digestion of plasma PrPc can be conducted without addition of SDS. We can now titer plasma and determine which PK condition is appropriate to digests all PrPc and no infectivity. We also identified the condition for plasma PrPres denaturation. We are currently developing the reagents and protocols for a "proof of principle" titration in plasma after PK digestion. This is the last step in the development of a complete plasma diagnostic assay. In a large, long term, "limiting dilution" titration of untreated, whole urine from scrapie infected hamsters, we have now conclusively shown that urine from TSE infected animals contains significant levels of infectivity and we will have a precise titer in another 6 months. Urine could thereby be an alternative substrate for disease detection. Bladder and kidney are also infectious.

15. SUBJECT TERMS
PrPres, Assay, Diagnostics, PMCA, ELISA, Blood, Urine

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Table of Contents

Introduction

Transmissible spongiform encephalopathy (TSE) diseases are fatal illnesses for which there is no cure or treatment. Individuals incubating TSE can transmit infectivity by blood transfusion. Three human-to-human transmissions of variant Creutzfeldt-Jakob disease were reported in the United Kingdom in the past two years[1-3]. TSE infectivity in blood was also demonstrated in natural and experimental animal models such hamster[4,5], mouse[6], and sheep[7]. This funding has supported the development of a prototype assay for TSE infection using hamsters infected with the 263K stain of scrapie.

Great efforts have been directed towards the development of a pre-mortem preclinical diagnostic test using blood as the test material. Several companies and groups have reported assay platforms and technologies with high sensitivity and specificity to detect PrP^{res} in the blood of human or animals affected with TSE diseases[8-13]. The field of TSE diagnosis is moving fast and new claims appear frequently in the news. None of these tests have been independently validated, and none are yet commercialized. For the most part the developers have used animal models to assess assay sensitivity, robustness and reproducibility. Among the most noteworthy assays are, the Prionics test with a PrPres specific monoclonal antibody 15B3[8] and the Soto's assay[14]. None of the assays commercially developed depend on proteinase K (PK) digestion for discrimination between normal PrP^{c} and abnormal, disease-specific PrP^{sc} (or PrP^{res}). Each developer has a proprietary method that supposedly detects only PrP^{res} and no PrP^{c}. In general these assays are conducted with relatively small volumes of plasma (< 1 ml). All diagnostic tests use ELISA or similar methods for the final detection of the PrP^{res} signal. One exception is the Soto assay utilizing the protein misfolding cyclic amplification assay (PMCA) with Western blot analysis as the final detection method[14].

The aim of this work is to develop a pre-mortem diagnostic test capable of identifying individuals incubating TSE disease in the asymptomatic phase. The target test material is blood and the protein to be detected is PrP^{res}, the biochemical marker for TSE infections. Our approach was to systematically evaluate each step of the assay protocol to assess its limit of detection and when possible to confirm the Origen Analyzer signal with Western blot analysis. We differ in this with the rest of the groups developing a diagnostic.

We also proposed to attempt to resolve the conflicting reports on the presence or absence of TSE infectivity in urine by applying the sensitive and precise method of "limiting dilution" titration that we developed for quantitating TSE infectivity in blood to urine. We have conclusively demonstrated the presence of low but significant levels of TSE infectivity in urine and will have a

final measurement of the concentration six months from now.

Body

In this report as in all previous ones, the specific aims numbering was modified to reflect the elimination of the first specific aim from the original proposal.

Specific aim 1 – Task 1

In the last report we described an experiment in which urine from scrapie infected hamsters was inoculated intracerebrally back into the same species. A cohort of animals was also inoculated with urine from age match uninoculated hamsters. This study was conducted to try to resolve the various inconsistencies in the reports on the presence of TSE infectivity in urine[15-18]. Urine is a desirable test material for a TSE diagnostic because of easy accessibility in relatively large quantities.

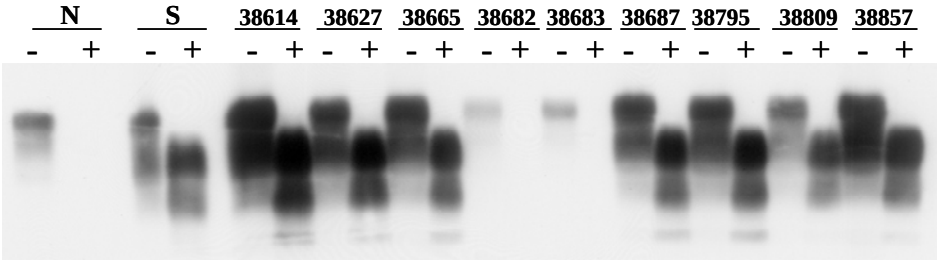
Our first priority was to assess the level of infectivity in urine. In brief, we inoculated 300 animals with urine collected from scrapie infected hamsters diluted 1:3 before inoculation. The dilution was necessary to reduce the toxicity of undiluted urine. Normal urine was not toxic and could be inoculated undiluted into 40 hamsters. Each animal was inoculated intracerebrally under deep anesthesia with 50 μl sample. In all 15 mls of a 1:3 dilution of the clinical sample, or a 5 ml equivalents of undiluted urine was inoculated. This is the volume that we have used successfully for precise measurements of the concentration of TSE infectivity in blood with the limit of detection at 0.2 infectious doses per milliliter. We used the end point dilution titration method to measure the infectivity in kidney and bladder tissues of clinically infected hamsters.

The results so far are described in tables 1 and 2 for urine and tissues titrations, respectively. Table 1 shows that at 357 days post inoculation 11 animals inoculated with urine from infected animals developed the disease. Every animal that died of scrapie was tested for the presence of PrP^{res} in the brain. Figure 1 shows the Western blot results of this analysis for a representative group. It is clear that these animals died of scrapie infection as indicated by the presence of large concentrations of PrP^{res} (+PK) in the brain. The Western Blot result demonstrates that the sick animals contracted scrapie rather than an unrelated disease due

Table 1 **357 days post inoculation**

Urine source	Volume ml	Animals inoculated	Scrapie animals	Titer
Infected hamsters	**4.87**	**292**	**11**	**2.3 ± 0.4**
Uninoculated hamsters	**2**	**40**	**0**	**0**

to the toxic effect of urine. None of the 40 animals inoculated with urine from normal animals developed infections. It is important to emphasize that the critical parameter in these titrations is not the number of animals inoculated but the volume of sample assayed. In the case of infected urine, 5 ml equivalents of undiluted sample were assayed while 2 ml of normal urine was inoculated (50 μl x 40).
Table 2 shows the results of the bladder and kidney titrations. The titers calculated with the Reed and Muench method[19] indicate much greater infectivity in both organs than we were expecting. Kidney was considered to be a minimally infected tissue. Bladder, to our knowledge, has not previously been tested for infectivity.
Figure 1



The tissue titrations will be completed at 365 days post inoculation. The urine study will continue to 540 days post inoculation. The distribution of incubation times of infections for samples with low infectivity such blood and urine spreads from 120 to 540 days post inoculation[5]. Earlier termination of the titration would underestimate the titer of the sample. The titer of the urine could increase by another 50% to two fold.

The implications for infectivity in urine are several and span different aspects of TSE diseases. First, urine could be used as an alternative to blood for TSE diagnosis. The infectivity levels are not much lower than that in infected plasma (5-10 ID/ml) (suggesting a possible source of the infectivity). Second, human urine is the source of therapeutical hormones used to treat female infertility. Risk assessment of this procedure may be needed. Third, if these data can be generalized to natural forms of TSE such as chronic wasting disease, bovine spongiform encephalopathies and scrapie, our results point to urine as a

Table 2 **343 days post inoculation**

Dilution	Bladder Tot/infect	Kidney Tot/Infect
Undiluted	19/19	4/4
1:2	8/8	20/20
1:5	8/8	8/8
1:10 (10-1)	4/4	8/8
10-2	4/4	4/4
10-3	4/2	4/1
10-4	4/1	4/0
10-5	4/0	4/0
Titer $\log_{10}ID_{50}/g$	5.5±0.5	5.0±0.3

possible source of environmental contamination and horizontal transmission in animals in the wild and in captivity. These data have already generated interest in the field and they will be presented for the first time at the Prion 2006 meeting in Turin, Italy in October.

Specific aim 2 – Task 2

As indicated in our previous report, we measured PrP concentration in normal hamster brain at 7.5±0.9 μg/g and PrP in infected brain at 57±9.6 μg/g. These measurements were conducted using the Origen Analyzer assay with two monoclonal antibodies. The pool of scrapie brain homogenate had been titered and the titer was 2.3 x 10^{10} ID_{50}/g tissue. Thus, we can calculate the ratio 4.6 x 10^{8} ID_{50}/μg PrP^{res}. Using 1 ID_{50} = 0.693 ID^{20} the brain titer can be converted to 3.2 x 10^{8} ID/μg. The blood titer is 10 ID/ml which corresponds to 31 fg/ml PrP^{res} at clinical phase, less at preclinical conditions. This point emphasizes that detection of PrP^{res} in small volumes of blood by an antibody-based assay is highly unlikely as typically the limit of detection of immuno-assays is in the order of nanograms of analytes. If our assumptions are correct the majority of assays commercially developed do not detect PrP^{res}. Again, the only exception is the PMCA assay. This conclusion does not imply that these assays are not discriminating normal from infected blood samples but they are doing so with the detection a protein other than PrP^{res} which may function as surrogate markers for infection. Alternatively, if the assumptions we used for our calculations are incorrect, and the concentration of PrP^{res} in plasma is much higher than that estimated from our work, we would expect much higher concentrations of infectivity in blood.

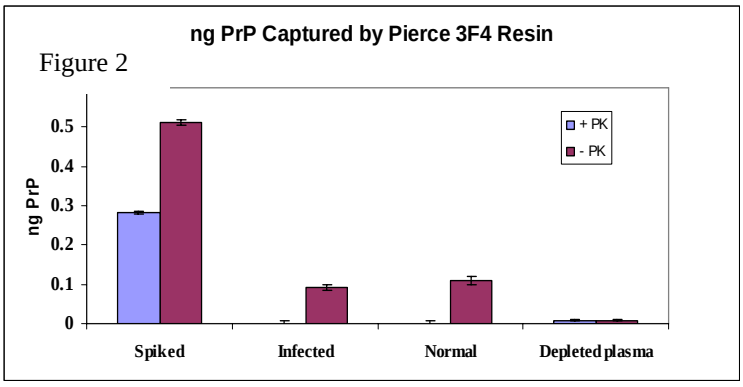


Figure 2

To increase the assay signal we incorporated a PrP^{res} concentration step (Pierce 3F4 resin) before the detection with Origen analyzer. Concentration is conducted by capturing PrP from undiluted plasma with an immune-affinity resin. The previous report extensively described this step and the current assay conditions. More recently, we applied the final assay to the detection of PrP^{res} in hamster plasma. In this test, four samples were prepared: scrapie brain homogenate spiked into PrP^{c}-depleted

hamster plasma (see previous report for the preparation of this plasma), PrP^{c}-depleted plasma, normal hamster plasma and scrapie infected hamster plasma. Each sample was tested in two conditions, with and without PK, for a total of eight samples. Each sample was mixed with the immuno-affinity resin to capture both PrP forms (PrP^{c} and PrP^{res}), the resin was eluted and the eluted material was assayed with the Origen analyzer. The results of this experiment are described in figure 2. The assay detected PrP spiked into plasma (higher signal without PK and lower signal with PK). No signal was detected with PrP^{c}-depleted normal hamster plasma as all PrP had already been removed. The normal hamster plasma also showed detectable signal without PK and no signal with PK as all PrP^{c} was digested. The infected hamster plasma showed PrP signal without PK and no signal with PK, thus no PrP^{res} signal could be detected from infected plasma. This study was repeated three times with the same results. There are several conclusions that can be drawn from this experiment. First, the assay is capable of detecting brain PrP^{res} spiked in plasma thus, it is a valid platform for a TSE diagnostic in blood. Second, under our conditions no plasma PrP^{res} could be detected although PrP^{c} in normal and infected plasma were detected. Because of technical constraints with the assay volume the largest volume of infected plasma tested was 100 μl. Thus, the data indicated that 100 μl plasma does not contain detectable PrP^{res}. We are investigating ways to increase the testable sample volume. Third, we do not know whether our PK digestion conditions also removed PrP^{sc} (see below). In conclusion, these results were very encouraging because they revealed an assay capable of capturing PrP^{res} in plasma and at the same time they highlighted the need for more information about the appropriate PK conditions for PrP^{sc} in plasma.

We have not conducted any work with Biotraces in this last period and no funding was drawn from the grant. The collaboration is still open but the work was put on hold until a better and more sensitive assay platform is developed and fully tested by Biotraces. The company is expected to open a laboratory at the University of Maryland at the Baltimore campus. When this happens we will be able to establish a more efficient collaboration with Biotraces' research and development staff.

Specific aim 3 – Tasks 2 and 3

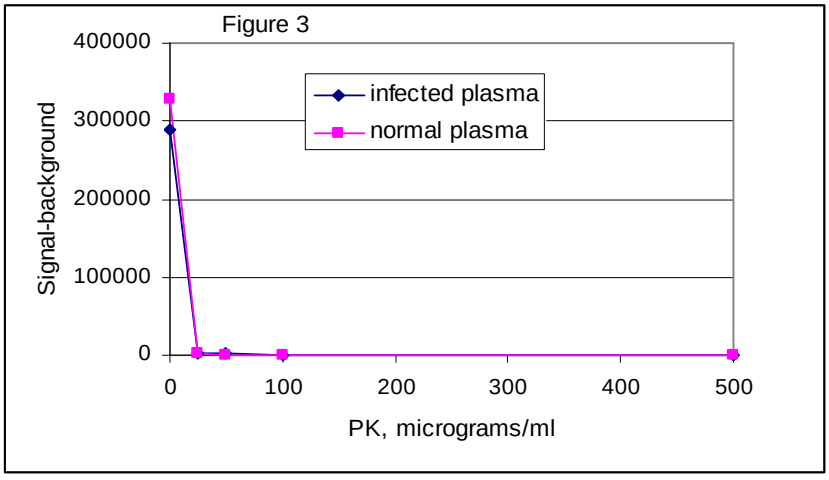
Major breakthroughs in the PK digestion step were obtained since the last report. The most important observation relates to the use of SDS in the digestion. We extensively investigated the effect of PK, SDS and brain spike concentrations in plasma as well as time and temperature reaction. We used brain derived PrP^{res} as surrogate for endogenous plasma PrP protein. We concluded that relatively

high concentrations of SDS (0.5%-1%) were necessary to completely digest brain spike PrP^{c} to reveal lower concentrations of PrP^{res}. Brain spikes required SDS for complete digestion of PrP^{c}. The level of removal that could be detected was > 99.9%. More recently, we began testing hamster plasma without the brain spike and discovered that SDS is not needed to remove endogenous PrP^{c} to the limit of detection. The level of removal after the immuno affinity resin that could be demonstrated was >99%. The 10-fold difference with the brain spike PK digestion is due to the higher level of signal from brain compared to plasma. Omission of SDS from PK digestion of plasma is important for two reasons. The first reason is that milder PK conditions are less likely to digest endogenous plasma PrP^{res} and second, the PK treated plasma will be inoculated into hamsters for titration and this step is incompatible with high concentrations of SDS.

The second important observation is that following PK digestion plasma can be effectively denatured with 1% SDS. This is 2-fold less detergent than in the previous protocol and allows for less dilution in successive step. The denaturation is needed to expose the epitope for PrP recognition by the immuno affinity resin. This modification of the protocol practically doubles the sample size and thus increases the signal.

Specific aim 3 – Tasks 1 and 4

The PK conditions for plasma PrP^{res} cannot be determined as PrP^{res} cannot be detected. Therefore, lack of PrP^{res} signal in 100 µl of infected plasma demonstrated in figure 2 could be due to inappropriate PK conditions. We proposed to measure the infectivity remaining after PK digestion of infected plasma using the animal bioassay. Assuming correlation between infectivity and PrP^{res}, this study will indicate the correct PK conditions to digest PrP^{c} and not PrP^{res}. PrP^{res} will be tracked by titration of endogenous infectivity. In the experiment, plasma will be digested under different PK concentrations and the digested plasma will be inoculated intracerebrally into hamsters. Reduction of infectivity will indicate that PrP^{res} was reduced by PK digestion. The first step was to determine the concentrations of PK and that of its inhibitor. Figure 3 shows the titration results for PK concentration. 50



μg/ml PK lowered the PrP^c signal close to the limit of detection, but 100 and 500 μg/ml consistently pushed the signal to below the background level. In the animal bioassay the PK treated plasma is inoculated into animals. We anticipated PK to be toxic when intracerebrally inoculated and therefore we investigated PMSF and pefabloc as two PK inhibitors. PMSF was tested but it was eliminated because it was too toxic. Pefabloc was less toxic. The final studies indicated that 500 μg/ml PK was partially inhibited with 150 mM pefabloc. However, this concentration of inhibitor could not be inoculated into animals (see below). Lower concentrations of PK require lower concentration of inhibitor. The preparative work for this study is almost complete and awaits results from toxicity studies.

Toxicity Studies

As part of the preparation for the titration of plasma in the bioassay, we determined the concentration of PK and of pefabloc tolerated by the animals (these data are not available in the literature). These studies concluded that the highest concentration of pefabloc that could be safely inoculated was 5 mM and that PK could be inoculated with pefabloc at all concentrations including 500 μg/ml. The animals are still under observation for long term effect of the inoculum. If the animals are healthy for another month, we will consider the toxicity study complete and we will be ready for the experiment.

Key research accomplishments

- Urine from infected animals is infectious
- A measurement of the concentration of infectivity in urine is near completion
- Kidney and bladder from infected hamsters have relatively high titers of infectivity.
- The level of PrP^{res} in the infected hamster plasma was calculated based on our assay and found to be ~ 30 fg/ml.
- We have defined the details of the plasma based assay in hamster infected with scrapie.
- We have optimized and refined the PK conditions for digestion of endogenous plasma PrP^c.
- We have completed toxicity studies for PK digestion of hamster infected plasma.

Reportable outcomes

In the previous report we included a manuscript to be submitted for publication. We had omitted to include funding from this grant in the acknowledgements. We regret this error and we are going to

correct it in the new version of the same manuscript that has been updated with our newer data for submission.

Conclusions

The research program is on schedule. We did not encountered unexpected problems that require revision of the proposed studies. The assay developed has been investigated with endogenous infected hamster plasma and showed no PrP^{res} signal. We are investigating the possibility that PK used to remove plasma PrP^{c} may have digested PrP^{res}. This investigation requires assaying the infectivity in plasma post PK digestion.

Our studies of urine have now conclusively demonstrated that this biological fluid contains low but detectable levels of infectivity that would be a suitable target for assay. This is a major result that deserves further investigations.

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Appendices

none